Developing Live Biotherapeutics
to target Immuno-Oncology

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1st Microbiome Movement – Oncology Response
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Live Biotherapeutics

- Single-strain commensal bacteria encapsulated for oral delivery
- Highly favourable toxicity/side-effect profile
- Accelerated preclinical development and early in-patient data

Industry leader with differentiated approach

- 4D pharma is an integrated platform & product company
- Mechanistic approach focused on function: targeting and addressing known disease pathways via effector molecules
- Sector-leading IP estate; more than 550 granted patents
- Multiple value inflection points; proof-of-concept clinical data on the horizon

Breakthrough class of medicines with potential to change the way we treat disease
FOCUS ON FUNCTIONALITY: MicroRx® PLATFORM

**Isolation**
- Significant culturomics expertise
- Broad coverage and diversity
- Previously unisolated organisms

**Product development**
- Integrated scale-up and optimization
- Strain-specific fermentation and formulation

**Strain engineering**
- Gene disruption/deletion
- Recombinant protein expression
- Anti-sense RNA gene silencing
- CRISPR-Cas9 gene editing

**Host-response assays**
- Targeted immune screening approaches
- Suite of host cells, spheroids/organoids
- Host receptors/MAMPs

**Genome mining**
- WGS and genome mining
- Comparative genomics and bioinformatics

**Metabolomics**
- Metabolomic fractionation
- Exhaustive profiling of effector molecules

**Proteomics**
- Cell surface shaving (surfaceome)
- Characterisation of secretome
- Targeted and driven by host signalling data
Successful immunotherapy requires two components:
- Activation of anti-tumour immunity
- Reversal of immunosuppression

Microbiome-derived LBPs could target more than one of the steps of the cancer-immune cycle.
SCREENING FOR IMMUNOSTIMULATORY LBP CANDIDATES

The MicroRx® platform employs a multi-faceted screening approach using combined in vitro and in vivo methods to identify strains with potent immunostimulatory profiles.

- Increase CD8⁺ and CD4⁺ T cell differentiation, reduce Treg differentiation
- Activation of antigen-presenting cells
- Induction of specific cytokine signatures across multiple human cell lines
- Translation to in vivo immune cell populations, systemic and tissue-specific
- Identification of effector molecules – metabolomics, genome mining, genetic manipulation, phenotypic assays
- Focus on function – e.g. LBPs with potent MAMP-signalling properties, LBPs with specific metabolic signatures affecting HDAC inhibition
Programme:
MRx0518 in Immuno-Oncology
MRx0518
• Gram-positive, motile, anaerobic bacterium of the *Enterococcus* genus
• Selected for immunostimulatory host response profile, relevant for immunotherapy

**Efficacy in vivo**
• Monotherapy: reduction of tumour volume in different syngeneic cancer models
• Combination: boosts efficacy of checkpoint inhibition

**Mechanism of action**
• Increases tumour CD8+/Treg ratio
• Immune stimulation through action of bacterial flagellin on TLR5

**Ongoing clinical studies**
• Phase Ib neoadjuvant biomarker study (UK)
• Phase I/II combination study with anti-PD-1 (US)
MRx0518 MONOTHERAPY IN SYNGENEIC TUMOUR MODELS

MRx0518 was tested across a range of different tumour models with varying degrees of immunogenicity.

### Cell line | Cancer type | Mouse strain | SC/OT | ICI sensitivity
--- | --- | --- | --- | ---
EMT6 | Breast carcinoma | BALB/C | SC | T/C <42% 42%<T/C<80% T/C >80%
RENCA | Kidney adenocarcinoma | BALB/C | SC | -
LLC1 | Lung carcinoma | C57Bl/6 | SC | CTLA-4 PD-1 PD-L1

#### Table 1: Tumour models

- **Tumour Volume**: Analysis of tumour growth over time.
- **Cytokine analysis**: Measurement of cytokine levels in the blood.
- **Transcriptomics (NanoString)**: Gene expression profiling.
- **Histopathology**: Examination of tissue samples.
- **Metabolomics**: Study of small molecules in the gut.
- **Inflammatory mediators**: Analysis of inflammatory markers in the blood.
- **Histopathology / immunohistochemistry**: Examination of tissue samples with staining.

#### Figures

- **Tumour**: Graph showing mean tumour volume over days post-tumour induction.
- **Microbiome**: Microbial composition analysis.
- **Blood**: Cytokine analysis.
- **Gut**: Transcriptomics analysis.

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Yu et al., 2018
MRx0518 MONOTHERAPY INHIBITS TUMOUR GROWTH IN DIFFERENT CANCER TYPES

MRx0518 showed efficacy in syngeneic tumour models of breast, kidney and lung cancer

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumour volume (Mean±sem)</th>
<th>T/C (%) at D28 vs Untreated</th>
<th>T/C (%) at D28 vs Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1010.88 ± 282.98</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1134.43 ± 362.03</td>
<td>106.37</td>
<td>-</td>
</tr>
<tr>
<td>MRx0518</td>
<td>572.93 ± 77.61</td>
<td>55.29</td>
<td>51.97</td>
</tr>
<tr>
<td>Anti-CTLA-4</td>
<td>14.33 ± 8.57</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumour volume (Mean±sem)</th>
<th>T/C (%) at D22 vs Untreated</th>
<th>T/C (%) at D22 vs Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>483.36 ± 70.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle</td>
<td>356.93 ± 78.86</td>
<td>49.77</td>
<td>-</td>
</tr>
<tr>
<td>MRx0518</td>
<td>243.29 ± 50.53</td>
<td>40.06</td>
<td>80.49</td>
</tr>
<tr>
<td>Anti-CTLA-4 + Anti-PD-L1</td>
<td>41.79 ± 28.52</td>
<td>0.00</td>
<td>0.00</td>
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</tbody>
</table>

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The effects of MRx0518 on immune cell types across different tissue sites were investigated

- Increased NK cells, T cells and cytotoxic cells in the tumour
- Increases NK cells, T cells and cytotoxic cells in the ascending colon
- Few changes in ileal immune populations
MRx0518 SELECTION – STRONG IMMUNOSTIMULATORY PROFILE IN VITRO

MRx0518 induces a strong innate immune response in vitro

- MRx0518 increases the production of a cytokine/chemokine signature that includes IL-8, IFN-γ, IL-6, TNF-α, IL-1β, IL-23, CCL20, CXCL1, CXCL3, CXCL9 and CXCL10
MRx0518 induces a strong CD4\(^+\) and CD8\(^+\) adaptive immune response \textit{in vitro}

### PBMC co-culture assay

<table>
<thead>
<tr>
<th></th>
<th>CD8(^+) cells</th>
<th>CD4(^+) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD8(^+)CD25(^+) (of CD8(^+) cells)</td>
<td><strong>0.0001</strong></td>
<td></td>
</tr>
<tr>
<td>% IFN(^\gamma) (of CD8(^+) cells)</td>
<td><strong>0.0001</strong></td>
<td></td>
</tr>
<tr>
<td>% CD4(^+) (of CD4(^+) cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IFN(^\gamma) (of CD4(^+) cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td><img src="chart1.png" alt="Bar Chart" /></td>
<td><img src="chart2.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>MRx0518</td>
<td><img src="chart3.png" alt="Bar Chart" /></td>
<td><img src="chart4.png" alt="Bar Chart" /></td>
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</tbody>
</table>

### Treg differentiation assay

<table>
<thead>
<tr>
<th></th>
<th>CD8(^+) FoxP3(^+) (of CD4(^+) cell population)</th>
<th>CD25(^+)IL-10(^+) (of CD4(^+) cell population)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28, IL-2 and TGF-(\beta)</td>
<td><strong>0.0001</strong></td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>% CD8(^+) FoxP3(^+) (of CD4(^+) cell population)</td>
<td><img src="chart5.png" alt="Bar Chart" /></td>
<td><img src="chart6.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>% CD25(^+)IL-10(^+) (of CD4(^+) cell population)</td>
<td><img src="chart7.png" alt="Bar Chart" /></td>
<td><img src="chart8.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>Untreated</td>
<td><img src="chart9.png" alt="Bar Chart" /></td>
<td><img src="chart10.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>MRx0518</td>
<td><img src="chart11.png" alt="Bar Chart" /></td>
<td><img src="chart12.png" alt="Bar Chart" /></td>
</tr>
</tbody>
</table>

- MRx0518 increases % CD8\(^+\) T cells and activated IFN\(^+\) CD8\(^+\) T cells
- MRx0518 increases % CD4\(^+\) T cells and activated IFN\(^+\) Th1 CD4\(^+\)T cells
- MRx0518 reduces differentiation of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Tregs
MRx0518 UPREGULATES INNATE IMMUNE CELL SUBSETS IN MICE

MRx0518 induces non-conventional T cell subsets in unchallenged mice

Systemic immune cells specifically upregulated by MRx0518

- Mice dosed with MRx0518 show increased frequency of systemic immune cell populations associated with anti-tumour immunity
Mechanism of Action –
Bacterial Effector Molecules
Identification of immunostimulatory factors of LBPs: integrated host-microbe approach

Host response assays
- Which host receptor(s) and pathways are stimulated?
- Which immune populations/mediators are induced and in which direction?
- What is the active bacterial fraction(s)?

Genome mining
- Whole genome sequencing and mining
- Transcriptional analysis of effector genes
- Comparative genomics
- ID potential MAMPs

Molecular tools
- Antigen over-expression
- Gene inactivation

Surfacome/Secretome
- Targets of interest related to host signalling

Immunomodulatory molecule ID
- Cell wall
- Cell-surface shaving
- CFS
- Surface Proteins
- Secreted Proteins

Discovery-based target indicators
- Genus/species of interest
- Immunomodulatory molecule ID

• Which host receptor(s) and pathways are stimulated?
• Which immune populations/mediators are induced and in which direction?
• What is the active bacterial fraction(s)?
IMMUNOSTIMULATORY FACTORS OF MRx0518

Immunostimulatory factors of MRx0518: identification of potential MAMPs

Whole genome sequencing and genome mining

- 3.13 Mb chromosome
- 3 plasmids (3-43 kb)
- 3,035 coding genes
- 2 predicted phage regions

Identification of potential MAMPs and host-interaction factors:

- Flagellin
- Adhesins
- Bile salt hydrolase

Surfacome/Secretome analysis

Relative abundance (PSM) | Role in host-interactions
--- | ---
Flagellin | 16 57 | Immunostimulation, adhesion to mucin
Heat-shock protein GroEL | 39 25 | Binding to mucin
GAPDH | 9 5 | Binding to mucin
Enolase | 63 52 | Binding to human plasminogen
Chaperone protein DnaK | 39 34 | Binding to human plasminogen
Eff-Tu | 143 38 | Binding to mucin, IL-8 stimulation
Choloylglycine hydrolase | 8 3 | Bile resistance, binding to plasminogen

Other molecules

- Secreted ATP
- Extracellular DNA
MRx0518 – IDENTIFICATION OF IMMUNOSTIMULATORY EFFECTORS

**Activation of NF-κB**

MRx0518 supernatant strongly activates NF-κB

- NF-κB activation is abolished by trypsin treatment

**Activation of TLR5**

MRx0518 supernatant strongly activates TLR5

- TLR5 activation is abolished by trypsin treatment

**Activation of TLR9**

MRx0518 supernatant strongly activates TLR9

- TLR9 activation is abolished by DNase and trypsin treatment
FLAGELLIN IS RESPONSIBLE FOR MRx0518 TLR5 ACTIVATION AND IL-8 PRODUCTION

Overall strategy

MRx0518 flagellin activates TLR5 and induces IL-8 secretion

TLR5 activation in response to SN
- Little to no TLR5 activation or IL-8 production when flagellin gene is knocked out
- MRx0518 induces higher TLR5 activation and IL-8 induction than the reference strain
- MRx518 stimulatory effects abolished by flagellin inactivation

Dose-response with recombinant flagellins
- Purified recombinant flagellins activate TLR5
- MRx0518 flagellin is more potent than the reference flagellin at low concentrations

MRx0518 flagellin is more potent than DSM100110 flagellin

Lauté-Caly et al., Sci Rep 2019

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MRx0518 AND DSM100110 EXPRESS DIFFERENT FLAGELLINS

MRx0518 and DSM100110 both produce functional, but divergent, flagella

Genotypic and phenotypic characterization

- Highest level of sequence divergence between MRx0518 and DSM100110 FliC sequences located in central variable region
- Regions known to be critical for TLR5 binding in other bacterial species were conserved between both strains

Characterization of additional motile strains

- Selected motile TLR5-activating strains induce IL-8 production in IECs
- Strongest TLR5 activation does not correlate with increased motility or IL-8 production – impact of variations in flagellin sequence?
TLR5 and Flagellin in Cancer: Supporting Literature

- Expression and activation of TLR5-associated pathways is elevated in breast carcinomas.
- Salmonella flagellin activation of TLR5 in breast cancer cells resulted in local cytokine release and inhibition of cell proliferation.
- A Salmonella strain expressing Vibrio vulnificus flagellin had tumour-suppressive effects in a colon cancer model.
- The engineered strain also decreased metastasis.
Programme:
MRx1299 in Immuno-Oncology
**MRx1299**

- Gram-negative, non-motile, anaerobic bacterium *Megasphaera massiliensis* species within the *Veillonellaceae* family
- Selected for immunostimulatory host response profile and metabolic signature

**Effects *in vitro* and *in vivo***

- Increases *in vitro* cytokine production and CD8+/Treg ratio
- Reduces clonogenic survival of different cancer cell lines
- Reduces tumour growth by adoptive cell transfer in syngeneic cancer models

**Mechanism of action**

- HDAC inhibition, potentially through action of SCFAs pentanoate and/or butyrate
- Enhances anti-tumor activity of CD8+ cytotoxic T cells

*Padmanabhan et al., Standards in Genomic Sciences (2013)*
OPPORTUNITIES FOR TARGETING HISTONE DEACETYLASE INHIBITION

- Histone deacetylase inhibitors induce gene expression changes through modulating the acetylation/deacetylation of histones and/or non-histone proteins such as transcription factors.

- Class I HDACs in particular have been linked to the development and growth of a range of cancers. We have focused on this activity as a target for anti-cancer candidates.

- HDAC inhibition may lead to re-recognition of the tumour by the immune system and down-modulation of immune-suppressive elements in the tumour microenvironment.

<table>
<thead>
<tr>
<th>HDACi in tumour cells</th>
<th>HDACi in immune cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>• (Immunogenic) cell death</td>
<td>• Activation of cytotoxic cells</td>
</tr>
<tr>
<td>• Upregulation of MHC proteins</td>
<td>• Suppression of regulatory cells, such as MDSCs and Tregs</td>
</tr>
</tbody>
</table>

Haematological malignancies seem to be sensitive to HDAC inhibitors, and pan-HDACi have given favourable results in a small set of patients with selected haematological diseases.

Approval of two structurally distinct HDACis - SAHA (vorinostat, Zolinza™) and FK228 (romidepsin, Istodax™).
TARGETING HISTONE DEACETYLASE INHIBITION BY MRx1299

*Megasphaera massiliensis* inhibits Class I HDACs through the production of SCFAs

- Class I HDAC isoforms HDACs 2 and 3 are inhibited by *Megasphaera massiliensis* MRx1299
- HDACs are more potently inhibited by MRx1299 than by equivalent concentrations of butyrate
- MRx1299 produces butyrate, and is a rare but prolific producer of pentanoate and hexanoate, both in monoculture and in a complex microbial community
- Pentanoate specifically has potent HDAC1 and HDAC2 inhibitory activity

Yuille et al., PLoS ONE 2018

Simmi: consortium of 17 human gut bacteria developed to model core metabolic cross-feeding functions of the human gut microbiota.

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MRx1299 INCREASES HISTONE ACETYLATION IN MELANOMA CELLS AND INDUCES CLONOGENIC CELL DEATH

MRx1299 increased acetylated H3 and H4 nuclear staining in melanoma (and CRC) cell lines, and this acetylation corresponds to reduced clonogenic growth.
MEGASPHAERA MASSILIENSIS INDUCES CD8+ T CELL-MEDIATED ANTI-TUMOUR IMMUNITY

*M. massiliensis* induces anti-tumour immune functionality

- Secretion of TNF-α by CTLs was increased after treatment with *M. massiliensis*-derived supernatants

- *M. massiliensis* increased the frequency of IFN-γ+TNF-α+CD8+ T cells

- CTLs pretreated with *M. massiliensis* and adoptively transferred into mice had increased capacity to infiltrate the tumours, produce effector cytokines and eradicate B16OVA cells as compared to control CTLs

- The anti-tumorigenic effects of *M. massiliensis* have been directly linked to histone acetylation in CD4+ and CD8+ cells at CTL-characteristic loci
MRx1299-induced immune activation was investigated in different cell types

- MRx1299 induces a cytokine/chemokine signature that includes IL-6, IL-22, IL-10, TNF-α, CXCL2, CXCL10, CCL3, CCL4 and CCL5, and increases the CD8/Treg ratio \textit{in vitro}
4D PHARMA: ADVANCING MULTIPLE IMMUNO-ONCOLOGY CANDIDATES WITH DIFFERENT MECHANISMS OF ACTION

**MRx0518**

*Species*
*Enterococcus gallinarum*

**In vivo efficacy**
- Increases tumour immune infiltration
- Increases microbiome diversity
- Reduces tumour growth in multiple tumour types

**Mechanism of Action**
- Flagellin TLR agonism

**Clinical Development**
- Phase I, monotherapy biomarker study in treatment naïve patients awaiting surgical resection of solid tumours, enrolling
- Phase I/II, combination with pembrolizumab in solid tumours (NSCLC, renal, melanoma, bladder), enrolling
- Additional studies planned

**MRx1299**

*Species*
*Megasphaera massiliensis*

**In vitro effects**
- Increases cytokine production and CD8+/Treg ratio
- Reduces clonogenic survival in multiple cancer cell lines

**In vivo efficacy**
- Reduces tumour growth by adoptive cell transfer in syngeneic cancer models

**Mechanism of Action**
- SCFAs pentanoate and butyrate inhibit HDAC

**Future Development**
- Studies of efficacy in pre-clinical models of additional tumours types ongoing
- CMC development and scale-up ongoing
developing science
delivering therapies